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An Immunologic Study on Age-related Macular Degeneration

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ABSTRACT: Forty-one patients with age-related macular degeneration (AMD) were detected for serum autoantibodies against normal human retinal protein by means of Western immunoblot analysis. Twenty-seven out of the 41 patients showed positive response, with a rate of 66 per cent. The positive rate of antiretinal antibody in the AMD patients was significantly higher than that in normal controls (18%) and in patients with other retinal diseases (24%) ($p < 0.0005$). These antiretinal antibodies from the AMD patients partly reacted with the retinal protein of molecular weight between 28 and 32 Kd, partly with Mr of 38 to 42 Kd, 48 to 52 Kd, 62 to 65 Kd or 110 to 130 Kd. Of them, the antiretinal antibody against the protein with Mr of 28 to 32 Kd in the AMD patients was higher than in normal controls ($p < 0.05$). Two or more antibodies were found in the serum from AMD patients, showing a significant difference between the patients and the controls ($p < 0.005$). The results indicated that in the occurrence of AMD and/or during its developing process there were inflammation and immunological response, involving antibodies against retinal proteins of various molecular weight. *Eye Science* 1993;9:113-120.

Key Words: age-related macular degeneration, anti-retinal antibody, Western immunoblot, immunology maculopathy

Introduction

Age-related macular degeneration (AMD) is now recognized to be one of the important ocular diseases causing blindness and affecting the elderly population all over the world. The etiology and pathogenesis of AMD are still unclear, although much work has been done on its epidemiology, pathology, serology, immu-

nology and etc.^[1] AMD was firstly regarded as an aged changes and degeneration related to age, resulted from a failure of retinal pigment epithelium (RPE).^[2] Metabolic disturbance which might play an important role in the disease was found in the elapsing years.^[3] In recent years, however, some results of immunologic studies indicate that AMD is possibly associated with inflammation. Penfold et al (1985) and others studied its pathology and discovered that many inflammatory cells and immunocompetent cells involved the lesions on eyes with AMD.^[4-7] Grossniklaus et al (1992) stained the subretinal neovascularized membranes obtained from the AMD patients by immunohistochemistry and histochemistry and found that the components of

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the extracellular matrix of the membrane are similar to those of the granulation tissues. They also found fibronectin in the interphotoreceptor cell matrix, which is normally devoid of it, and in diffuse drusen the fibronectin showed a high concentration.^[8,9] Penfold and collaborators (1990) disclosed several kinds of autoantibodies, including antiretinal antibody present in sera from the AMD patients.^[10] Gurne and colleagues (1991) demonstrated the presence of antiretinal antibody as well.^[11] In the present study, we investigated whether anti-retinal autoantibodies might be present in the disease process and with which retinal protein the antibody reacted. The sera of 41 patients were tested by means of Western immunoblotting and compared with healthy aged persons.

Materials and Methods

Subjects and preliminary procedures

Forty-one patients with AMD (29 men and 12 women), ranging in age from 50 to 83 years with a mean age of 63.8 years, were included in the study. All were diagnosed in Macula Clinic of Zhongshan Ophthalmic Center, Sun Yat-Sen University of Medical Sciences. They underwent a complete ophthalmologic examination, including fundus color photography fluorescein angiography and visual function assessment. Twenty-two of the patients had wet form and 19 had dry one. Thirty-two cases showed bilateral lesions. Control subjects were 17 healthy, visually unimpaired persons (7 men and 10 women), ranging in age from 51 to 78 years with a mean age of 58.6 years, and showing normal appearance of fundi. Blood samples were collected from the subjects, their sera were obtained by centrifugation and stored in aliquotes at -40°C before use. Additionally, sera from patients with the following retinal diseases were acquired: 14 patients with retinitis pigmentosa (RP), 5 with idiopathic preretinal membranes (PRM), 4 with central serous chorioretinopathy (CSC) and 2 with acute retinal necrosis (ARN). The eye globes were obtained from healthy adult men with sudden death by an accident within 15 hours post-mortem. The retinas were separated from the RPE and stored at -40°C before use.

Electrophoresis and immunoblots

Isolated retinas were homogenized in 10 mM Tris-buffer, pH 7.2, containing EDTA (1mM) and PMSF (1mM) by homogenizer. The protein concentration in the homogenate was 4 mg/ml (Lowry's method),^[12] which was then diluted with 4% SDS sample buffer containing 10% 2 Mercaptoethanol to yield a final concentration of 2 mg protein per ml of 2% SDS. The samples were centrifuged at 32,000 rpm for an hour, and separated by SDS-PAGE using 2.6% low cross-linked gels, formed as a 5 to 20% linear gradients: 4 μ g of protein was applied for every 1mm of gel well width and 20 μ g of SDS-PAGE protein molecular weight markers (17.5 to 94 KD) (provided by the Institute of Biochemistry, Academia Sinica, Shanghai, China) for a 5 mm width well at a side at the same time. After electrophoresis, the gels were electroblotted on nitrocellulose paper (Bio-Rad, Richmond, CA, USA) using the method of Towbin et al^[13] Transferred sheets with protein molecular markers were cut and stained with Coomassie brilliant blue R-250 and the remainings were incubated overnight with 5% bovine serum albumin to block nonspecific binding. After washing, the sheets were cut vertically, each 3 mm wide. Individual transblot strips were then reacted with diluted sera (1:200) from AMD patients and control subjects. After three intervening washes with Tris-buffer saline containing 0.3% Tween 20 (TTBS) at 10- minute intervals, the strips were reacted with biotinylated rabbit anti-human IgG (Dakopatts, DAKO, Carpinteria, CA, USA), washed three times again and reacted with HRP-Avidin (DAKO). After the final wash in TTBS, the blots were developed with the peroxidase substrate diaminobenzidine tetrahydrochloride. Molecular weight markers were used to estimate the molecular weight of the retinal proteins.

Results

The immunoreaction on Western blots of human retinal homogenate with serum antibodies from patients with AMD and controls was shown in figures 1 to 5. Under each original picture, we put a schematic diagram to illustrate it briefly

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and clearly. In the diagram, weak reactive and artifact bands were cleared away. The reactive intensity on blots was distinguished by duplicator. On many blots, staining bands were shown at several sites which implied several kinds of molecular weight retinal proteins

involved. The sera from subjects were considered to be positive when significant bands at Mr of 28 to 32 KD, 48 to 52 KD or 62 to 65 KD protein were revealed on blots. According to these criteria, 27 out of the 41 patients with AMD had positive reaction on immunoblotting.

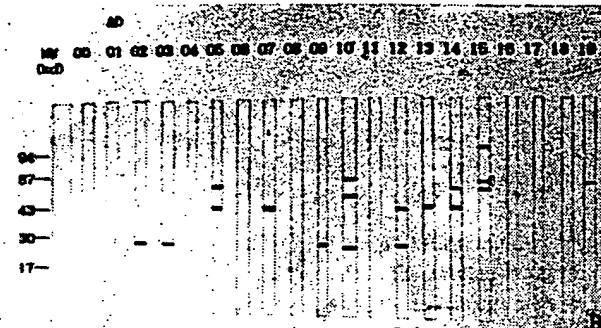
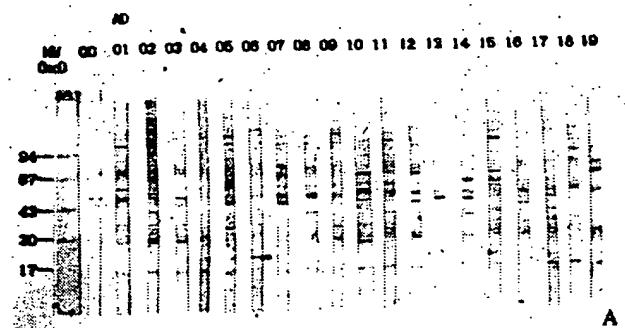


Fig 1. Immunoreaction on Western blots of human retinal homogenate with serum antibodies from patients with wet form of AMD. A: original picture; B: schematic diagram in which weak reaction and artifact bands were cleared away. Lane on the left: protein molecular weight standards. MW, molecular weight. 00: replacement of serum with TTBS; 01-22: patients with wet form of AMD. All dilutions of sera were 1:200.

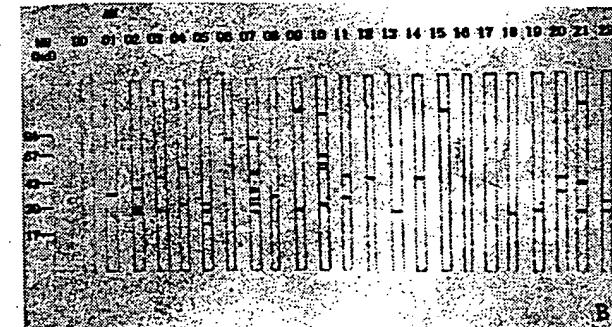
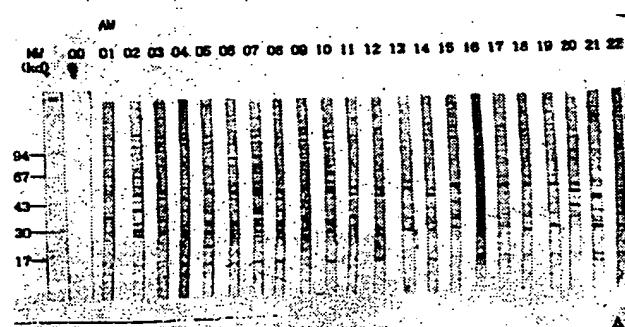


Fig 2. Immunoreaction on Western blots of human retinal homogenate with serum antibodies from patients with dry form of AMD. A: original picture; B: schematic diagram in which weak reaction and artifact bands were cleared away. Lane on the left: protein molecular weight standards. MW, molecular weight. 00: replacement of serum with TTBS; 01-22: patients with wet form of AMD. All dilutions of sera were 1:200.

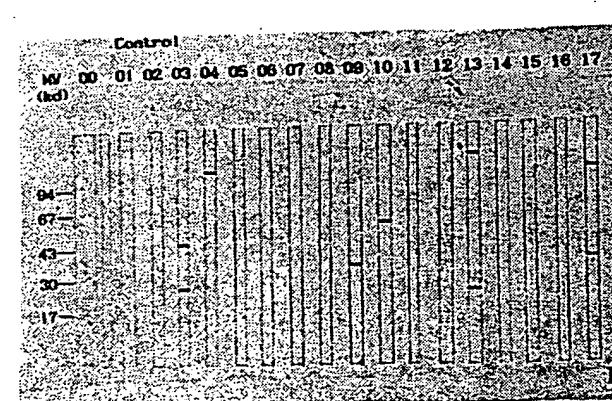
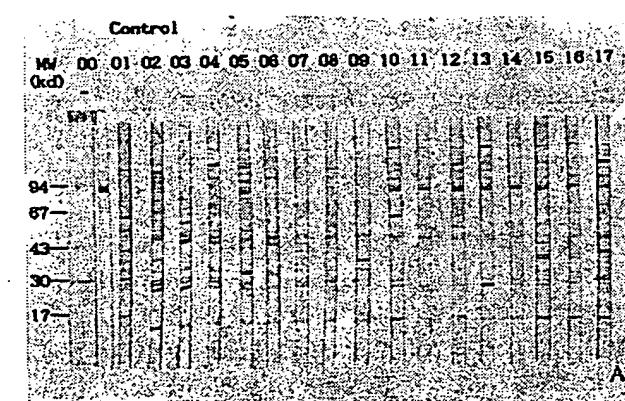


Fig 3. Immunoreaction on Western blots of human retinal homogenate with serum antibodies from normal controls. A: original picture; B: schematic diagram in which weak reaction and artifact bands were cleared away. Lane on the left: protein molecular weight standards. MW, molecular weight. 00: replacement of serum with TTBS; 01-17: normal subjects. All dilutions of sera were 1:200.

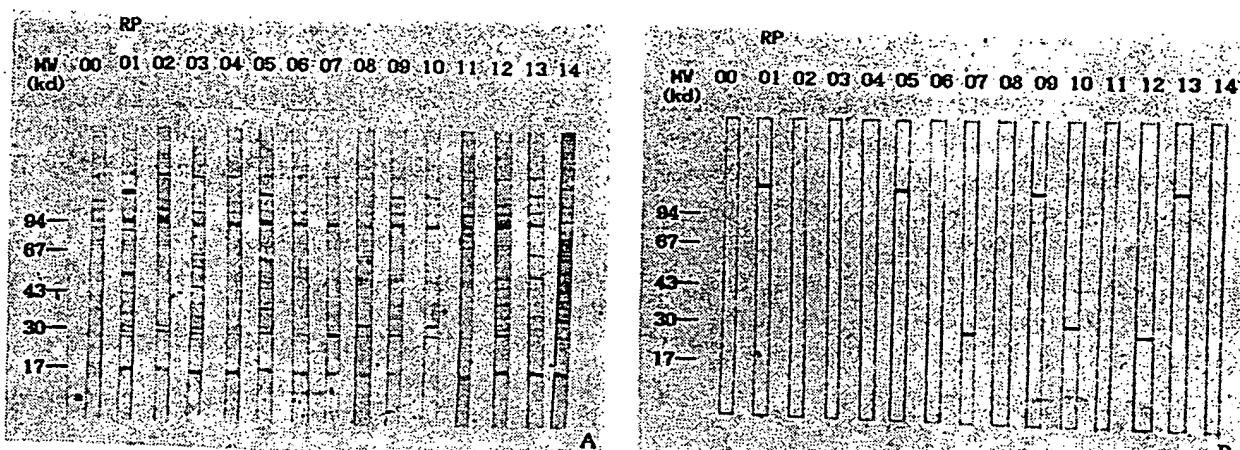


Fig 4. Immunoreaction on Western blots of human retinal homogenate with serum antibodies from patients with RP. A: original picture; B: schematic diagram in which weak reaction and artifact bands were cleared away. Lane on the left: protein molecular weight standards. MW, molecular weight. 00: replacement of serum with TTBS; 01-14: patients with RP. All dilutions of sera were 1:200.

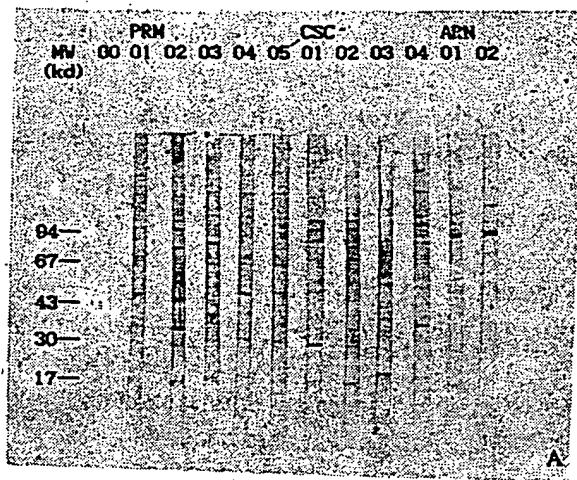


Fig 5. Immunoreaction on Western blots of human retinal homogenate with serum antibodies from patients with PRM, CSC, ARN. A: original picture; B: schematic diagram in which weak reaction and artifact bands were cleared away. Lane on the left: protein molecular weight standards. MW, molecular weight. 00: replacement of serum with TTBS; All dilutions of sera were 1:200.

Table 1. Antiretinal antibody incidence

Subjects	Total No.	Positive No.	Rate(%)
AMD	41	27	66
dry form	19	11	58
wet form	22	16	73
Normal	17	3	18
Other retinal diseases	25	6	24

with a positive rate of 66 per cent, whereas 3 out of the 17 normal healthy aged persons were positive. The incidence of antiretinal autoantibodies in AMD patients and controls was

seen in Tab 1. The expression of antibodies in AMD group was much more frequent than that in control group ($p < 0.0005$). No significant difference between wet and dry form was observed, although the sera from the patients with wet form showed more positive bands on blots ($p > 0.05$). However, the difference between the group of dry form of AMD patients and normal controls was significant ($p < 0.05$).

In AMD group, the sera of patients with dry form expressed the antiretinal antibody against Mr 28 to 32 Kd, 48 to 52 Kd or 62 to 65 Kd retinal protein, with a similar incidence, and often two antibodies in one case. However, the sera of patients with wet form produced

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antibodies which reacted with various retinal proteins and several kinds of antibodies always in every case. The class of retinal protein with which the autoantibody reacted was complicated, including the proteins involved in dry form as well as the proteins with Mr of 38 to 42 Kd, 88 Kd and 110 to 130 Kd (Fig 1 to 2). The antibodies against Mr 28 to 32 Kd, 48 to 52 Kd or 38 to 42 Kd retinal protein were seen more

often and usually two or more antibodies were simultaneously present in each case. The antiretinal antibodies responsible for molecular weight of retinal protein were tabulated (Tab 2). The incidence of antibody against retinal protein with Mr of 28 to 32 Kd in AMD patients was significantly higher than that in normal controls ($p < 0.05$). When sera were tested repeatedly, staining bands were found to be consistent.

Table 2. Antiretinal antibodies profiles

Subjects	28-32	38-42	48-52	62-65	88	110-130(Kd)
AMD	17	7	12	6	2	5
dry form	5	0	5	4	0	1
wet form	12	7	7	2	2	4
Normal	2	2	1	1	0	3
Other retinal diseases	5	0	2	1	2	4

Two or more antibodies against various retinal proteins in sera were present often in many AMD patients each but less in normal subjects and patients with other retinal diseases (See Tab 3.). The frequency of several antibodies in sera of the AMD patients was significantly higher than that in controls ($p < 0.005$).

Table 3. Frequency of antiretinal antibodies

Antibody No.	one	two	three or more
AMD (n=41)	15	10	5
Normal (n=17)	3	3	0
Other retinal diseases (n=25)	9	1	1

Discussion

Age-related macular degeneration was always recognized as an senile degeneration and involution related to age in the past.^[2] With age, retinal pigment epithelium (RPE) can not completely digest and get rid of its phagosomal particles, which results in an accumulation of lipofuscin granules within the RPE, a further

decrease in capabilities of engulfing and disposing of the outer segment discs of photoreceptors with an gradual increase of abnormal deposits in the internal and external sides of the RPE basement membrane (basal laminar deposits and drusen). Finally, the RPE and photoreceptors denatured and subretinal new vessels formed.^[14,15] Some investigators further discovered that light damages, lack of nutrition, system diseases and etc were associated with AMD after an epidemiologic study.^[1,3,15] They put forward an idea that AMD is a multifactor syndrome.^[11] Among the factors mentioned, the importance of chronic optic insult or light damages was especially emphasized. Some authors observed that retinal ultrastructure was affected after light exposure by experimental animal studies.^[13,15] They experimented on rhesus monkeys, baboons or rabbits respectively and gave them an acute or chronic exposure to light. The results were: retinal scar formation or exudation,^[3] vacuolation and disarrangement of the outer segment discs of the photoreceptors enlargement^[11] of the inner segment mitochondrias and reduction of the reduced ascorbate in retinas.^[3] The action of light also produced a number of toxic products such as free radicals which led to lipid peroxidation of the

outer segment membranes of photoreceptor.^[15] The RPE cells were unable to digest these abnormal molecules damaged by free radicals. In recent years, however, Penfold et al and others studied the pathology of AMD and disclosed many inflammatory cells and immunocompetent cells such as microphages, lymphocytes, plasma cells, giant cells etc. The results showed a significant increase in cell numbers between the disciform eyes and normal eyes, atrophic eyes or intermediate phases^[4] and normal eyes. Grossniklaus et al and Das et al stained the subretinal neovascularization membranes from the AMD patients with immunohistochemistry and histochemistry and found that the extracellular matrix of the membranes contained collagen of types I, II, IV and V; fibronectin; laminin; mucopolysaccharide; and lipid.^[8,9] These components were similar to those of the granulation tissue. They also found that fibronectin, which has a prominent activity causing inflammation, was present in the inner and outer collagenous zones of Bruch's membrane, and was also present in the interphotoreceptor cell matrix in membranes, a location normally devoid of fibronectin. Newsome and associates demonstrated a high concentration in diffuse drusen and speculated that high fibronectin concentration in diffuse drusen might in part be responsible for the association of diffuse drusen with subretinal neovascularization.^[16] Recently, the results from Penfold and associates revealed that there were several kinds of autoantibodies, including antiretinal antibody, in the sera from the AMD patients. Many sera produced antiretinal antibody staining located at astrocytes in the inner retinal layers and a little sera produced staining at the nerve fiber layer, ganglion cell and the photoreceptor cell body using the streptavidin-biotin fluorescent labelling technique.^[10] Gurne and coworkers^[11] confirmed the presence of antiretinal antibody, which reacted with a singlet or doublet protein of molecular weight between 58 and 62 Kd by Western blot and positively bound to the outer segments of the photoreceptors on intact retinas by indirect immunofluorescence. The present results indicated that antiretinal antibodies are present not only in the sera of the AMD patients,

but also have a high rate of incidence. There was a highly significant difference between the AMD patients and the normal controls. The present data further supported the idea that inflammatory components and immunologic responses are involved in the onset of AMD and its developing process.

Using Western immunoblotting techniques the present study not only detected antiretinal antibodies in the sera of patients with AMD but also revealed several kinds of antibodies against various molecular weight of retinal proteins. Many of the patients each had 2 or more kinds of antiretinal antibodies. In our data, 27 out of the 41 patients with AMD had antiretinal antibody, with a positive rate of 66 per cent. There was a highly significant difference between the patients and normal controls, with a positive rate of 18 per cent, and between the AMD patients and the patients with other retinal diseases, with a rate of 24 per cent ($p < 0.0005$). In the AMD group, although the patients with wet form had more antibodies than those with dry one, but there was no significant difference between them. The results from Gurne et al indicated that there was antiretinal antibody in the sera of the AMD patients with a incidence of 47 per cent, but no difference was found among AMD groups (drusen, atrophy and disciform lesions), and few antibodies were noted in the sera of the normal controls. A very low incidence of antiretinal antibody from their report may possibly be related to comparative small samples and young age of the control group. We also noticed in our experiment that, the younger the patients, the lower the positive rate of antibody. Penfold and coworkers found antiretinal antibodies were present in the sera from 128 patients with AMD, with a rate of 8 per cent. It showed the highest rate in the pigmentary disturbance group among the four distinct patterns, i. e., pigmentary disturbance, geographic trophy, disciform and drusen. The frequency of antiretinal antibodies in patients with various types of AMD was significantly higher than that in controls, with a rate of 20 per cent.^[10]

In present data, the AMD patients had antibody against retinal protein with Mr of 28 to 32 Kd with a higher frequency in their sera than

the antibody as Mr 48 to 62 Kd. In wet form 1 antibody ag whereas the incidence of proteins such as 62 to 66 Kd to 130 Kd in the serum of the sera more anti weights of seen in sera patients with the results one antiret singlet or between 58 and 62 of the AMD. Penfold of antiretin applied to i produced c sera dem patterns. antiretinal astrocytes antiretinal the nerve photorecep biotin flu results fr various an in the imn in the dise antibodies in our dat

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the antibodies against other retinal proteins such as Mr 48 to 52 Kd, 62 to 65 Kd and 110 to 130Kd. In the AMD groups, the patients with wet form had a high incidence of the serum antibody against Mr 28 to 32 Kd retinal protein, whereas the patients with dry form had similar incidence of the antibodies against various retinal proteins such as Mr 28 to 32 Kd, 48 to 52 Kd and 62 to 65 Kd. The antibody against Mr 110 to 130Kd retinal protein was always present in the serum of the patients with wet form. Most of the sera from AMD patients showed three or more antibodies against various molecular weights of retinal proteins, which were seldom seen in sera from the normal subjects and the patients with other retinal diseases. However, the results from Gurne et al indicated that only one antiretinal antibody which reacted with a singlet or doublet protein of molecular weight between 58 and 62 Kd, was present in the sera of the AMD patients.

Penfold et al^[10] observed five distinct patterns of antiretinal antibody staining, when sera were applied to intact human retinas. Most of the sera produced one staining pattern and some of the sera demonstrated more than one staining patterns. A lot of sera produced filamentous antiretinal antibody staining located at retinal astrocytes and some sera produced other antiretinal antibody staining patterns located at the nerve fiber layer, ganglion cell and the photoreceptor cell body using the streptavidin-biotin fluorescent labelling technique. The results from our experiment indicated that various antigens, not a single antigen, take part in the immunologic and inflammatory responses in the disease process. However, two or more antibodies in each of many cases were revealed in our data.

The sera from the AMD patients, as we also noticed, produced antiretinal antibody against Mr 48 to 52 Kd retinal protein with a rate of 29 per cent. This retinal protein is considered to be S-antigen, located at the cytoplasms of the receptors.^[11] The present results showed S-antigen might take part in the disease process. The nature of the antigen of other retinal proteins, especially Mr 28 to 32 KD protein, which induced the antibody in the highest rate in this disease, is not defined and deserves further

study.

Recent studies on retinitis pigmentosa (RP) and cone dystrophy for evaluating immune responses to retinal antigens suggested a possibility that immune system may contribute to the retinal degenerative process. The results from Chant et al^[12] showed antiretinal antibodies were present in sera of patients with RP (37%) and those with other eye diseases (33%) and in sera of controls (2%). Isashiki et al^[13] found antibodies against Mr 14KD retinal protein in sera from the patients with cone dystrophy. Our study confirmed the presence antiretinal antibodies in sera of RP patients, who often have one antibody only, and a much lower incidence compared to that in AMD.

Of course, although the presence of antiretinal antibodies in sera of patients dose not predicate that antibody formation as a primary factor in the disease process, circulating antibodies many aggravate a degenerative process initiated by other etiologic agents, thereby converting the normally protective immune system into a destructive one.

The positive rate of antiretinal antibody from the AMD patients was predominantly higher than that of normal controls from our results. In addition, the patients with wet form had a higher positive rate of antiretinal antibody than those with dry one. It seems that the antiretinal antibody increases as the disease progresses, which in turn causes the disease develop further. Because the antibody can cross the blood-retinal barrier (BRB),^[10] where AMD develops unilaterally, antiretinal autoantibodies may promote retinal degeneration in the fellow eye. However, whether these antiretinal antibodies originate from the early phase of the disease or during the developing phase? Can we speculate the occurrence, development or prognosis of the disease based upon the presence of antibodies in the serum? It needs further observation. It is worth mentioning that antibodies of the IgG and IgM class have been detected in the drusen from one donor eyes with AMD.^[14] Drusen are believed to be the most characteristics feature of AMD. It is not known whether those antibodies in drusen are consistent with the antibodies detected in our experiment.

It is not clear what causes the antiretinal

antibody formation. Some authors suspected the activation of pre-existing clones of antibody-producing B cells in this disease.^[11] It has been hypothesized that an aberrant equilibrium between idiotypic and anti-idiotypic antibodies may break the immunoregulatory barrier, yielding loss of self-tolerance and the induction of autoimmune disease.^[11] During the disease process we speculate, the components including antigens, which came from the indigestion of the substances from the receptors by the RPE, deposited in the inner and external side of the RPE basement membrane and attracted and activated the immunocompetent cells, thereby producing the antiretinal antibodies. These antibodies may result in a series of inflammation and immunologic responses which promote the disease process.

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